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14. ABSTRACT
Vitamin D may be associated with breast cancer (BC) of poor prognosis, particularly the triple-negative (TN) subtype, which may explain the strikingly high incidence of (TNBC) among African American (AA) women, because AAs have significantly lower vitamin D levels than European Americans (EAs). Pre-treatment 25OHD levels were available from 579 EA BC cases and 574 EA controls, and compared by tumor characteristics with control for covariates. In 547 AA and 381 EA BC patients and 461 AA and 382 EA controls, tagSNPs in VDR, CYP27B1 and CYP24A1 genes were typed. BC cases had lower 25OHD than controls. Among premenopausal women, serum 25OHD was lowest among TNBC cases. Every 10 ng/mL increase in 25OHD was associated with a 64% lower odds of having TNBC (OR=0.36, 95% CI=0.22-0.56). In AAs 4 VDR SNPs were associated with BC risk, including rs2239186 (p by permutation =0.03). Women with GG/GA had increased level of serum vitamin D, as well as lower risk of BC than those with AA genotype (OR=0.53, 95% CI=0.35-0.79). Two SNPs in CYP24A1 were associated with ER- cancer risk, and adding them into a base model including race, substantially reduced increased risk of ER- cancer with AA women. In conclusion, we found lower vitamin D levels in association with BC of poor prognosis, particularly TNBC among premenopausal women. The associations of SNPs in vitamin D-related genes differ by race, and may explain, in part, the higher risk of ER negative cancer in AA women.

15. SUBJECT TERMS
Breast cancer, aggressive characteristics, vitamin D, VDR, SNP, racial disparity

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INTRODUCTION

The proposed research project is to 1) examine serum 25-hydroxy vitamin D (25OHD) levels in association with breast cancer aggressive characteristics, and 2) examine the contribution of vitamin D and VDR polymorphisms to breast cancer racial disparity between African-American (AA) and European American (EA) women. The two objectives are addressed in a two-step approach using two different study populations. The first objective was examined among breast cancer patients enrolled in the DataBank and BioRepository (DBBR) at Roswell Park Cancer Institute; the second objective was nested in the Women's Circle of Health Study (WCHS), a large scale ongoing case-control study with both AA and EA women. By the end of the grant, we have completed both projects. The results from this study have produced two meeting abstracts, one published paper, and another manuscript in review, and have been used as a part of preliminary data in a NCI-funded R01 and a NCI-funded P01 grant.

BODY

<u>Project 1. Serum 25OHD levels and breast cancer aggressive characteristics.</u>

In case-control and case-series analyses, we examined serum concentrations of 25-hydroxyvitamin D (25OHD) in relation to breast cancer prognostic characteristics, including histologic grade, estrogen receptor (ER), and molecular subtypes defined by ER, progesterone receptor (PR) and HER2. Included were 579 women with incident breast cancer and 574 controls matched on age and time of blood draw who were enrolled in the Roswell Park Cancer Institute Data Bank and Biorepository (DBBR) from 2003 to 2008. Patients' clinical data, including tumor stage, histologic grade and ER, PR and HER2 status, were obtained from a clinical database maintained by the RPCI Breast Program, and supplemented with data from abstracted medical records and the RPCI Tumor Registry. Because IHC of CK 5/6 or EGFR is not routinely performed in pathology, we defined four molecular subtypes in our study based on ER, PR and HER2 as follows: luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), non-luminal HER2+ (ER-, PR- and HER2+), and triple negative (ER-, PR- and HER2-). Serum 25OHD levels were compared between cases and controls, as well as among patients by tumor characteristics, including stage, grade, ER status, and molecular subtypes, with adjustment for covariates.

Table 1.1 shows serum 25OHD concentrations according to selected demographic and lifestyle characteristics of the control population. Younger women tended to have higher 25OHD levels than older women, although the differences were not statistically significant. There were apparent seasonal variations of serum 25OHD concentrations, with a peak during summer season. Circulating 25OHD concentrations were inversely associated with BMI, and positively associated with physical activity. Women who had higher dietary vitamin D intake or took vitamin D supplements had higher circulating concentrations.

The medians of serum 25OHD concentrations in breast cancer cases and controls were 22.8 ng/mL and 26.2 ng/mL, respectively. A majority of the controls were either vitamin D deficient (26%) or insufficient (36%), and only 39% of controls had a sufficient level of 30 ng/mL or higher. The proportion of vitamin D deficiency was even higher in cases (39%), and only a small proportion of them were considered vitamin D sufficient (21%) ($p<0.001$). As shown in Table 1.2, compared to women who were vitamin D deficient, those with sufficient levels had a 64% reduction in odds of breast cancer (OR=0.36, 95% CI=0.26-0.50). Every 10 ng/mL incremental increase of 25OHD concentrations was associated with an estimated reduction of breast cancer odds by one third (OR=0.67, 95% CI=0.59-0.75), which was significant in both premenopausal and postmenopausal women.

When pre- and postmenopausal women with invasive breast cancer were considered together, there were no significant differences in serum 25OHD concentrations by histologic grade or ER status (data not shown). However, women with triple negative breast cancer had the lowest vitamin D concentrations among the 4 molecular subtypes after control for age, BMI and season of blood collection (least square mean ± standard error: 23.0 ± 0.5, 21.3 ± 1.3, 21.6 ± 1.6 and 19.9 ± 1.1 ng/mL for luminal A, luminal B, non-luminal HER2+ and triple negative subtypes, respectively, p=0.046). In addition, there was an inverse relationship between serum

25OHD concentrations and tumor stage (26.5 ± 1.0, 23.2 ± 0.5, 21.3 ± 0.7 and 21.9 ± 2.0 ng/mL for stage 0 [CIS], stage I, stage II/IIIA, and stage IIIB/IIIC/IV, respectively, p<0.001).

When stratifying by menopausal status, serum 25OHD levels did not differ by tumor characteristics among postmenopausal women, but there were notable differences among premenopausal women (Table 1.3). Those diagnosed with invasive breast cancer, especially late stage cancer, had significantly lower 25OHD concentrations than those with CIS (p<0.001). Among premenopausal women with invasive breast cancer, those who had high grade or ER negative cancer had lower serum 25OHD concentrations than those with high grade or ER positive cancer (p≤0.03). Moreover, premenopausal women diagnosed with triple negative cancer tumors had the lowest concentrations compared to those with the other three molecular subtypes (p=0.002).
In case-control analyses, ORs and 95% CIs of breast cancer by menopausal status and tumor prognostic characteristics are plotted in Figure 1.1. Among premenopausal women, those with 25OHD concentrations above the median had significantly reduced odds of grade III cancer (OR=0.46, 95% CI=0.29-0.74), ER negative cancer (OR=0.34, 95% CI=0.17-0.66), and triple negative cancer (OR=0.21, 95% CI=0.08-0.53). Using continuous vitamin D data, an incremental increase of 10 ng/mL 25OHD concentrations was associated with about two thirds reduction of odds of triple negative breast cancer (OR=0.36, 95% CI=0.22-0.56) (Figure 1.2). Among postmenopausal women, higher serum vitamin D levels were associated with reduced odds of breast cancer regardless of tumor characteristics.

In case-series analyses, high levels of serum 25OHD were less likely to be associated with premenopausal breast cancer with poor prognostic characteristics than low levels (grade III versus I/II, OR=0.45, 95% CI=0.22-0.91; ER negative versus positive, OR=0.48, 95% CI=0.21-0.93; triple negative versus luminal A subtype, OR=0.26, 95% CI=0.09-0.71) (Figure 1.3). Similar results were also found with a 10 ng/mL incremental increase of serum 25OHD concentrations (Figure 1.4). In contrast, there were no associations of 25OHD levels with cancer prognostic characteristics in parallel analyses among postmenopausal women (Figures 1.3 and 1.4).

<u>Project 2. The contribution of vitamin D and related polymorphisms to breast cancer racial disparity between AA and EA women.</u>

In the Women's Circle of Health Study (WCHS), we investigated SNPs in VDR and in key vitamin D metabolizing genes CYP27B1 and CYP24A1 in relation to breast cancer risk in AA and EA women. We first genotyped 122 SNPs in an extend VDR region in 70 healthy AA and 70 EA controls for tagSNP selection by TAGSter program. A total of 52 tags were selected to cover the LD in both the AA and EA populations. In addition, we selected 15 multi-population tagSNPs for CYP24A1 gene and 1 SNP for CYP27B1 gene based on HapMap data. These SNPs were then genotyped in cases and controls from WCHS by Illumina GoldenGate assays. Also included in the genotyping chip were 111 ancestry informative markers (AIMs) to control for genetic admixture. The average call rate was 96.9% per sample and 96.9% per SNP. SNPs with poor clustering or excessive heterozygosity (n=3) and samples with lower call rate than 85% were removed (n=20). Among 5% blind duplicates included in the assays, there were no discordant results. Proportion of European ancestry was estimated by STRUCTURE program and AAs with an estimate of over 0.85 (n=11) and EAs of below 0.15 (n=3) were excluded. As a result, 547 AA breast cancer cases and 461 controls and 381 EA cases and 382 controls, were included in the analysis. Cochran-Armitage test for trend were used for univariate SNP analysis, and LD block-based haplotype analysis were performed. Multiple comparison error was controlled by 10,000 permutations. Covariates controlled in the multivariate logistic regression models were age, proportion of European ancestry, family history of breast cancer, BMI, and education. Modification effects by dietary intake of vitamin D and calcium were tested and stratified analysis were performed. All analyses were conducted in AA and EA separately using PLINK program, and the snp.plotter R package was used to generate plots with univariate SNP p-values and LD map.

Descriptive characteristics. Table 2.1 summarizes the descriptive characteristics of the study population by self-reported race. The majority of the women were premenopausal at the time of cancer diagnosis (62%) or enrollment for controls (57%). Overall, AA women had higher BMI than EA women (31.3 vs 27.2 kg/m2), were less likely to have a college education or beyond (57.5% vs 82.0%), to take hormone replacement therapy after menopause (14.0% vs 24.1%), and to have family history of breast cancer in first-degree relatives (13.5% vs 22.4%) (all p<0.001). There were no significant case-control differences in AAs or EAs, except that in EA

women, cases were more likely than controls to have more years of education and positive family history of breast cancer (p≤0.001).

Serum levels of 25OHD. Among controls, serum levels of 25OHD were lower in AA than EA women (least squared means and standard error after controlling for age, BMI, and season of blood collection: 14.9 ± 0.5 vs. 21.4 ± 0.6 ng/ml, p<0.001). As shown in Figure 2.1, the rate of frank deficiency (<10 ng/ml) was almost six-fold higher in AA than EA women (34.3% vs 5.9%) and, among AA women, the proportion of estimated African ancestry was inversely related to serum vitamin D levels. We categorized AA women by proportion of African ancestry (<85%, 85-94%, and ≥95%) and found that women with the lowest African ancestry had the highest serum 25OHD levels (15.5 ng/ml) while those with the greatest African ancestry (≥95%) had the lowest levels (13.7 ng/ml) (p=0.07). When testing correlations between SNPs and serum 25OHD levels in AA and EA women, the minor alleles of VDR SNP rs2239186 were significantly associated with increased levels of 25OHD in AAs. For the AA, AG and GG genotypes, the mean and standard deviation of serum 25OHD were 13.5 ± 6.5, 16.3 ± 8.7 and 21.2 ± 12.2 ng/ml, respectively (p=0.006). However, the differences were not significant in EA women (data not shown).

Associations between genetic variants and breast cancer risk by self-reported race. In addition to circulating vitamin D levels, there were also racial differences in genetic variants. Of the 65 SNPs genotyped, 51 (79%) displayed significantly different allele frequencies by self-reported race (p<0.05), including 12 SNPs that were the rare variant in one group (AA or EA) but the common allele in the other group. LD in VDR and CYP24A1 also displayed different patterns between AAs and EAs as shown in Figures 2.2 and 2.3. Figures 2.2 and 2.3 also show unadjusted p-values for associations between single SNPs and breast cancer risk. In AA women, four SNPs in VDR [rs12721364, rs2239186, rs886441 and rs11568820 (Cdx2)] but none in CYP24A1 were associated with breast cancer risk at a nominal significance level of 0.05 (Figures 2.2a and 2.3a). The association of VDR rs2239186 remained significant after correction for multiple testing (p=0.03). In EA women, 2 SNPs in VDR [rs11608702 and rs7975332 (Apa1)] and 3 SNPs in CYP24A1 (rs912505, rs3787555 and rs2244719) were associated with breast cancer risk (p < 0.05) (Figures 2.2b and 2.3b), but did not remain significant after controlling for multiple comparisons (data not shown). There were no associations between the SNP in CYP27B1 and breast cancer risk in either EA or AA women.

Table 2.2 shows ORs and 95% CIs for 4 SNPs (rs11608702, rs12721364, rs2239186 and rs11568820) in VDR and 2 SNPs (rs912505 and rs3787555) in CYP24A1 which had differential associations between AA and EA women (p for interaction by race ≤0.10) after adjustment for age, proportion of European ancestry, body mass index, family history of breast cancer, and education. In AA women, the combined GG and AG genotypes of rs2239186, which remained significant after correction for multiple testing and was also related to increased levels of 25OHD, was associated with an almost halving of risk of breast cancer compared to homozygotes for A alleles (OR=0.53, 95% CI=0.35-0.79, p-trend for the G allele=0.001). Among AA women, there was also reduced risk associated with VDR rs12721364 (OR=0.53, 95% CI, 0.31-0.79, p=0.01), and marginally increased risk with SNP rs11568820 (Cdx2) (OR for AA genotypes 1.94, 95% CI 1.01-3.74, p=0.04). Among EA women, although the VDR 'at risk' G allele for rs2239186 was more common in EA women, it was not associated with breast cancer risk (OR=0.85, 95% CI=0.62-1.17), nor were VDR rs12721364 SNPs. There were increases in risk by the VDR SNP rs11608702 and significant decreases in risk by two CYP24A1 variants rs912505 and rs3787555; however, these did not remain significant after correction for multiple testing. Results from haplotype analysis were consistent with those from single SNP analysis for VDR rs2239186. Among AA women, a G-G-G haplotype consisting of this SNP and two neighboring variants was associated with a decreased risk of breast cancer after adjusting for multiple testing (OR=0.55, 95% CI=0.38-0.81, p=0.04) (Table 2.3). Among EA women, similar results were also found for haplotypes containing rs11608702 in VDR and haplotypes containing rs3787555 in CYP24A1. The commonly studied haplotype in the 3' UTR of VDR consisting of Taq1, Apa1 and Bsm1 was not associated with breast cancer risk in AA women, but a modest decreased risk was observed in EA women, with marginal significance (OR=0.82, 95% CI=0.67-1.02).

Estrogen receptor negative breast cancer and CYP24A1 variants. Stratification by ER status revealed associations that were not observed in the overall analysis, with the majority of findings observed only for ER-negative breast cancer. Although VDR rs10783218 was marginally associated with a twofold increased risk of ER-positive breast cancer among EA women, and VDR rs3819545 was associated with decreased risk of ER negative breast cancer, several SNPs in CYP24A1 were highly significantly associated with risk of ER-negative

breast cancer. Importantly, results differed markedly between AA and EA women (p for interaction ≤0.10). For example, CYP24A1 rs27622941 was associated with more than a twofold increased risk of ER-negative breast cancer among AA women (OR=2.62, 95% CI=1.38-4.98), with no effect in EAs. Conversely, CYP24A1 rs2209314 was associated with almost threefold decreased risk of ER-negative breast cancer in EA women (OR=0.38, 95% CI=0.20-0.73), with no associations among AA women.

To determine whether these SNPs contributed to the observed higher risk of ER-negative breast cancer in AA women compared to EA women, a base model containing self-reported race and other covariates was developed (Table 2.4). The base model showed an increased risk of ER-negative cancer associated with AA race (OR=1.53, 95% CI=1.06-2.22). The 8 SNPs that showed significant interactions with race were tested in the base model. After backward selection, the 2 CYP24A1 SNPs shown above, including rs2209314, and rs2762941 , remained significant in the final model, reducing the risk associated with AA race by 22% and rendering it non-significant (OR=1.20, 95% CI=0.80-1.79).

Lastly, there were significant interactions for two SNPs in VDR with menopausal status. The increased risk associated with rs886441 in AA women was restricted to premenopausal women (OR=2.27, 95% CI=1.32-3.90), and the increased risk associated with rs7975232 (Apa1) in EA women was restricted to post-menopausal women (OR=2.24, 95% CI=1.19-4.21).

KEY RESEARCH AND TRAINING ACCOMPLISHMENTS

- We obtained additional pretreatment serum samples and data from 579 newly diagnosed breast cancer patients and 574 health controls from DBBR. Our analysis showed that serum 25-hydroxyvitamin D levels were lower in patients with breast cancer than controls in both premenopausal and postmenopausal women. When we further examined the relationship with breast cancer characteristics, we found premenopausal women with high vitamin D levels were less likely to have highly aggressive breast cancer, particularly the triple negative subtype, than those with low vitamin D levels.

- We measured serum 25OHD levels in 242 AA and 187 EA healthy women enrolled in the WCHS. Our results showed that vitamin D levels were much lower in AA women than in EA women. The difference remained after controlling for BMI and age. The prevalence of severe vitamin D deficiency was almost 6-fold higher in AA than in EA women.

- We genotyped 65 multi-population tagSNPs in VDR, CYP27B1 and CYP24A1, as well as 110 ancestry informative markers in a total of 1,771 AA and EA breast cancer cases and controls. We found that within AA women, serum vitamin D levels were inversely correlated with the proportion of African ancestry.

- We analyzed the genotype data in relation to breast cancer risk separately by race, and the found the associations between SNPs in vitamin D-related genes and breast cancer risk were race-specific. One SNP in VDR gene was associated with higher vitamin D levels and lower risk of breast cancer in AA women.

- When stratifying the analyses by ER status, we found that 2 SNPs in CYP24A1 substantially reduced the increased risk of ER-negative breast cancer in AA women than in EA women.

- In 2009, I finished my PhD degree based in part on the training projects funded by DOD.

REPORTABLE OUTCOMES

- A manuscript tilted “Pretreatment serum concentrations of 25-hydroxyvitamin D and breast cancer prognostic characteristics: A case-control and a case-series study” has been published on PLoS One in 2011.

- A manuscript entitled “Variants in the vitamin D pathway, serum levels of vitamin D, and estrogen receptor negative breast cancer among African-American women” is in preparation for submission.

- An abstract entitled “Common genetic variations in VDR, CYP27B1 and CYP24A1 genes and breast cancer risk in African American and European American women in the Women’s Circle of Health Study” was submitted to the AACR 2011 annual meeting, and was selected for a Scholar-in-training award.

- An abstract entitled “Vitamin D and breast cancer in African American and European American women” was submitted to the DOD BRCP 2011 Meeting, and was selected as a highlighted abstracted for press release.
- Results generate from this study was used as a part of preliminary data in a competitive renewal of an R01 grant the Pathways Study to investigate at vitamin D with breast cancer prognosis. This study has been recently funded by NCI (R01 CA105271, PI: Kushi). Our results were also used as a part of preliminary data in a P01 grant to investigate evolutionary factors including vitamin D and pigmentation in relation to triple negative breast cancer in AA women. This grant was also successfully funded by NCI (P01 CA151135, PIs: Ambrosone, Palmer, Millikan).

CONCLUSION

To conclude, we found premenopausal women with cancer of high aggressive characteristics including triple negative subtype, had much low serum 2-OHD levels than those with less aggressive cancers, indicating that vitamin D may prevent or delay breast cancer progression and reduce risk of breast cancer of high aggressive characteristics. A significant reduced risk of breast cancer was found in postmenopausal women with high vitamin D levels but there was no difference in vitamin D levels by tumor characteristics. The fact that the majority of the breast cancer patients are vitamin D deficient or insufficient at diagnosis confirms the epidemic vitamin D deficiency in the US, especially in breast cancer patients who may benefit from increasing vitamin D levels. In a second study, we found the associations of vitamin D-related genetic polymorphisms had differential associations with breast cancer between AA and EA women, and two SNPs in CYP24A1 explained in part the higher risk of ER-negative breast cancer in AA women than in EA women. This data provide the first evidence that vitamin D and related genetic variations may contribute to breast cancer racial disparity.

So what: Our results show vitamin D may prevent breast progression and reduce the racial disparity of breast cancer between African American and European American women. If the results are further validated in a prevention trial, young African American women particularly those at high risk of developing breast cancer shall take vitamin D to prevent breast cancer occurrence and progression.

REFERENCES

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SUPPORTING DATA

Tables and figures

Table 1.1. Serum 25-hydroxyvitamin D concentrations by demographic and lifestyle characteristics among healthy controls

Characteristics	N (%)[1]	Serum 25OHD, median (IQR), ng/ml	P-value[2]
Age, year			0.56
<50	202 (35.2)	28.3 (19.8-36.3)	
50-59	169 (29.4)	27.0 (19.2-33.4)	
60-69	127 (22.1)	26.8 (19.4-32.3)	
≥70	76 (13.2)	26.7 (19.8-33.5)	
Season of blood collection			<0.001
Spring (Mar-May)	99 (17.2)	25.7 (15.9-33.2)	
Summer (Jun-Aug)	175 (30.5)	30.5 (22.9-36.9)	
Fall (Sep-Nov)	135 (23.5)	25.2 (19.5-32.8)	
Winter (Dec-Feb)	165 (28.7)	24.7 (16.5-31.8)	
BMI, kg/m^2			<0.001
<25.0	184 (33.0)	30.7 (24.4-38.8)	
25.0-29.9	198 (35.5)	27.5 (20.7-33.2)	
≥30.0	175 (31.4)	21.6 (15.4-28.2)	
Physical activity			<0.001
More active	264 (46.2)	29.3 (22.2-36.5)	
Normal	198 (34.5)	24.3 (18.8-31.9)	
Less active	110 (19.2)	25.1 (16.1-33.2)	
Dietary vitamin D			0.003
Q1 (<42 IU/day)	146 (25.4)	24.0 (17.8-32.2)	
Q2 (42-147 IU/day)	134 (23.3)	27.3 (20.6-36.8)	
Q3 (148-329 IU/day)	142 (24.7)	27.6 (20.6-32.8)	
Q4 (≥330 IU/day)	152 (26.5)	27.3 (21.0-37.6)	

Supplementary vitamin D			<0.001
Yes	259 (45.1)	28.1 (22.2-35.0)	
No	315 (54.9)	24.9 (16.6-33.0)	

Footnote: [1] For some characteristics, the numbers did not add up to the totals due to missing data. [2] P-values were derived from Wilcoxon rank test for variables with two levels and Kruskal-Wallis test for variables with more than two levels. Abbreviation: IQR, interquartile range.

Table 1.2. Odds ratios and 95% confidence intervals for breast cancer by serum 25-hydroxyvitamin D levels

Serum 25OHD levels[1]	All			Premenopausal			Postmenopausal		
	case n (%)	control n (%)	OR (95% CI)[3]	case n (%)	control n (%)	OR (95% CI)[3]	case n (%)	control n (%)	OR (95% CI)[3]
Deficient	223 (39)	148 (26)	1.00	84 (34)	63 (26)	1.00	139 (42)	85 (26)	1.00
Insufficient	232 (40)	205 (36)	0.73 (0.55-0.97)	101 (41)	84 (34)	0.85 (0.53-1.36)	131 (39)	121 (37)	0.66 (0.46-0.96)
Sufficient	124 (21)	221 (39)	0.36 (0.26-0.50)	60 (25)	98 (40)	0.51 (0.30-0.85)	64 (19)	123 (37)	0.31 (0.20-0.47)
P-value for trend			<0.001			0.01			<0.001
Continuous per 10 ng/mL increment[2]	579	574	0.67 (0.59-0.75)	245	245	0.76 (0.63-0.91)	334	329	0.61 (0.52-0.72)

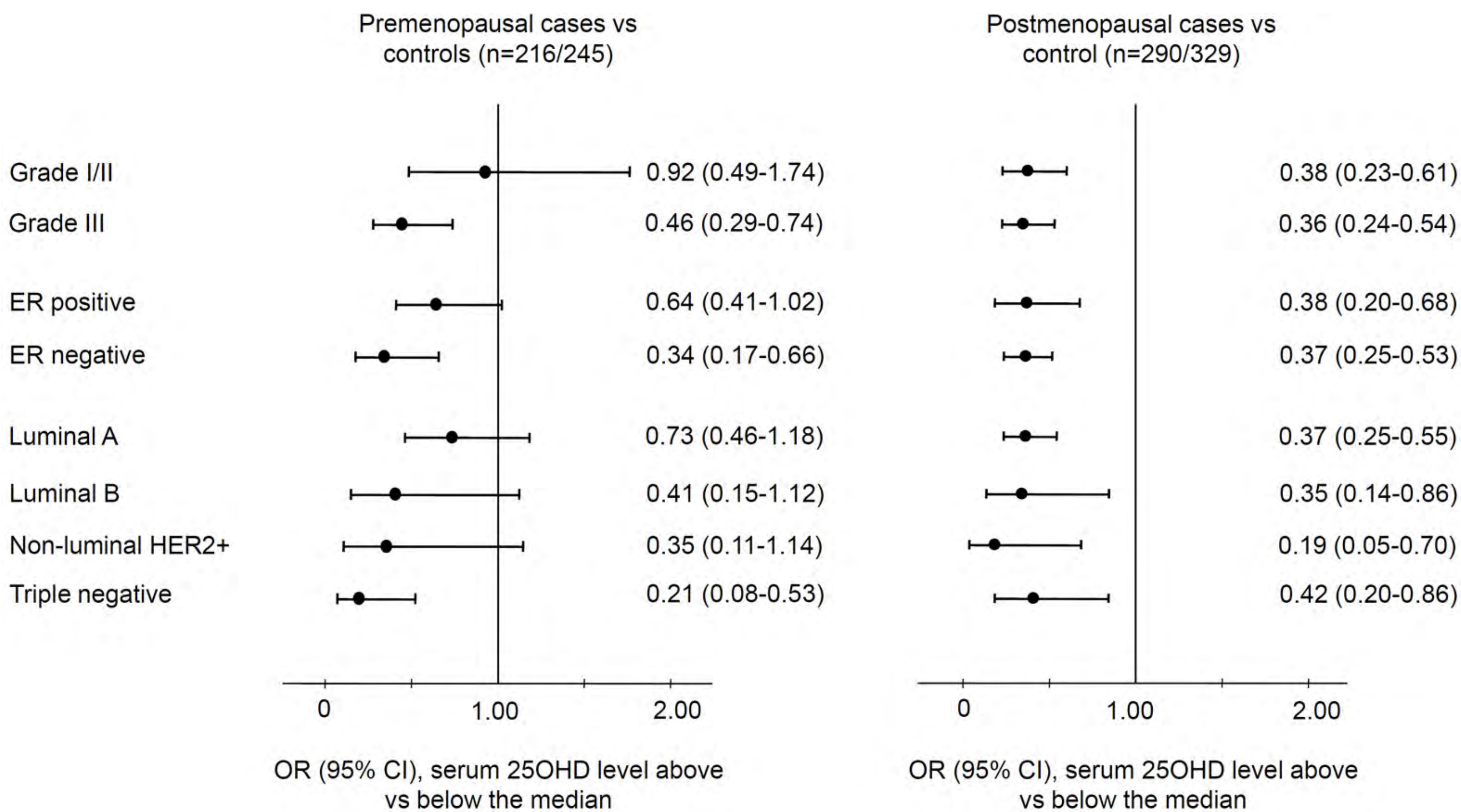
Footnote: [1]The three levels were defined as follows: deficient, <20.0 ng/mL; insufficient, 20.0-29.9 ng/mL; sufficient, ≥30.0 ng/mL. [2]Serum 25-hydroxyvitamin D (25OHD) concentrations were adjusted by the week of blood collection time in a year by locally weighted multinomial regression. [3]Odds ratios (OR) ad 95% confidence intervals (CI) were adjusted for age and BMI, and for the categorical levels of 25OHD, season of blood collection was also adjusted in the model. Further adjustment for physical activity did not significantly change the results (data not shown).

Table 1.3. Serum 25-hydroxyvitamin D concentrations by prognostic characteristics in premenopausal and postmenopausal women diagnosed with breast cancer

Tumor characteristics	All (n=579)	Premenopausal women (n=245)			Postmenopausal women (n=334)		
	N (%)[1]	N (%)[1]	mean ± se[2], ng/mL	P-value	N (%)[1]	mean ± se[2], ng/mL	P-value
Tumor stage				<0.001			0.23
In situ	86 (15)	42 (17)	28.9 ± 1.4		44 (13)	24.8 ± 1.4	
I	292 (51)	95 (39)	24.8 ± 0.9		197 (59)	22.3 ± 0.7	
II/IIIA	179 (31)	96 (39)	21.3 ± 1.0		83 (25)	21.4 ± 1.0	
IIIB/IIIC/IV	20 (3)	11 (5)	20.0 ± 2.7		9 (3)	24.4 ± 3.0	
Histologic grade				0.005			0.81
I/II	166 (35)	56 (29)	26.0 ± 1.3		110 (40)	21.9 ± 0.8	
III	305 (65)	137 (71)	21.6 ± 0.8		168 (60)	22.1 ± 0.7	
ER status				0.03			0.76
Positive	372 (76)	147 (73)	23.7 ± 0.8		225 (79)	22.1 ± 0.6	
Negative	115 (24)	55 (27)	20.2 ± 1.3		60 (21)	21.7 ± 1.2	
Molecular subtype				0.002			0.92
Luminal A	330 (69)	129 (64)	24.5 ± 0.8		201 (71)	22.2 ± 0.6	
Luminal B	49 (10)	23 (11)	21.2 ± 1.9		26 (9)	21.1 ± 1.7	
Non-luminal HER2+	32 (6)	15 (7)	21.7 ± 2.5		17 (6)	21.2 ± 2.2	
Triple negative	74 (15)	34 (17)	17.5 ± 1.6		40 (14)	21.8 ± 1.4	

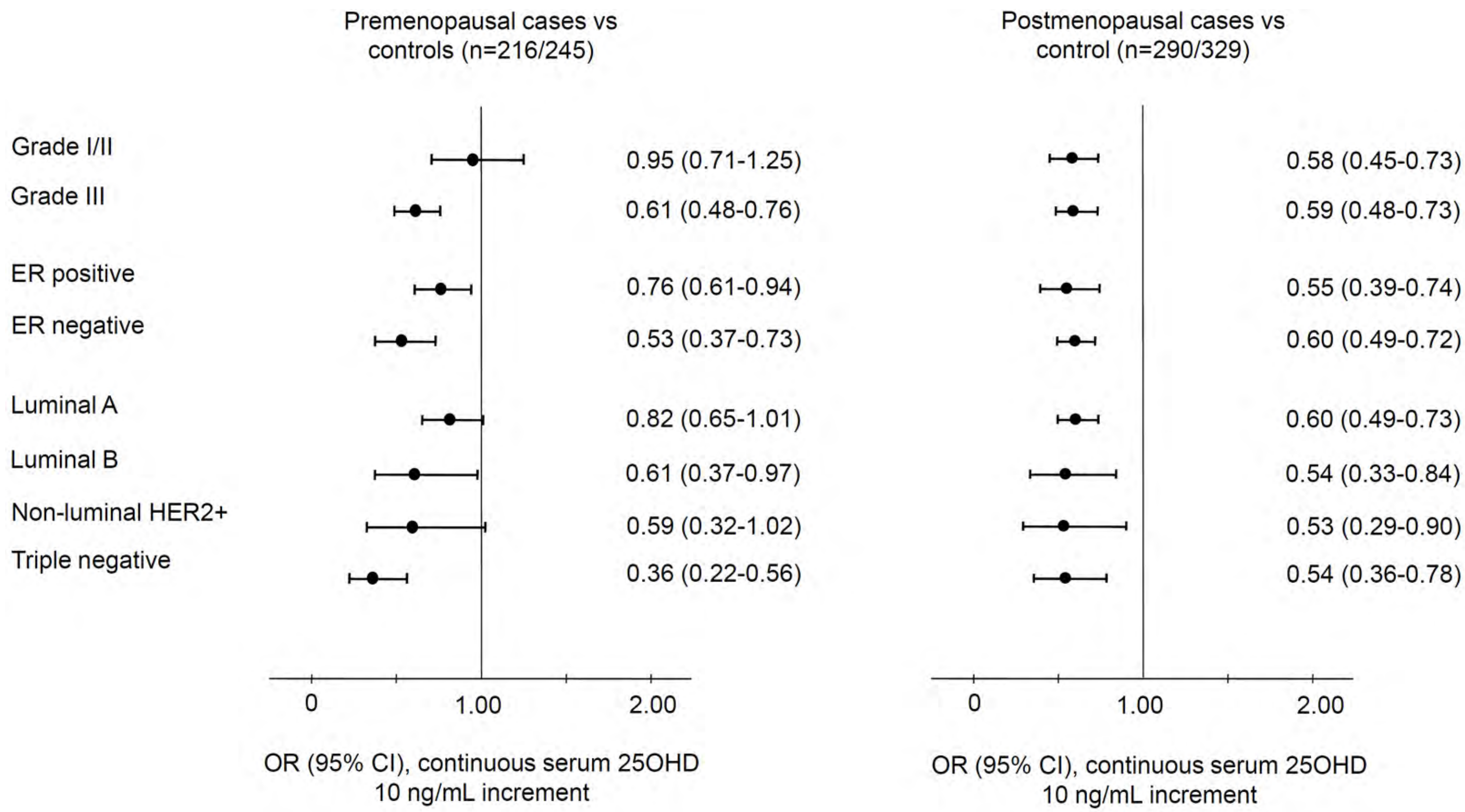
Footnote: [1]Two patients with tumor stage not evaluable (TX) were excluded from analysis of stage. For the analysis of histologic grade, ER status and molecular subtype, women with carcinoma *in situ* (n=86) were excluded. The numbers do not add up to the total due to missing data: histologic grade (missing n=22 or 4%), ER status (missing n=6 or 1%), and molecular subtype (missing n=8 or 2%). [2]Least square mean and standard error (se) were adjusted for age, season of blood collection, and body mass index in linear regression models. Additional adjustment for physical activity did not significantly change the results (data not shown).

Figure 1.1. Case-control analysis of breast cancer risk by prognostic characteristics with serum 25-hydroxyvitamin D levels in premenopausal and postmenopausal women



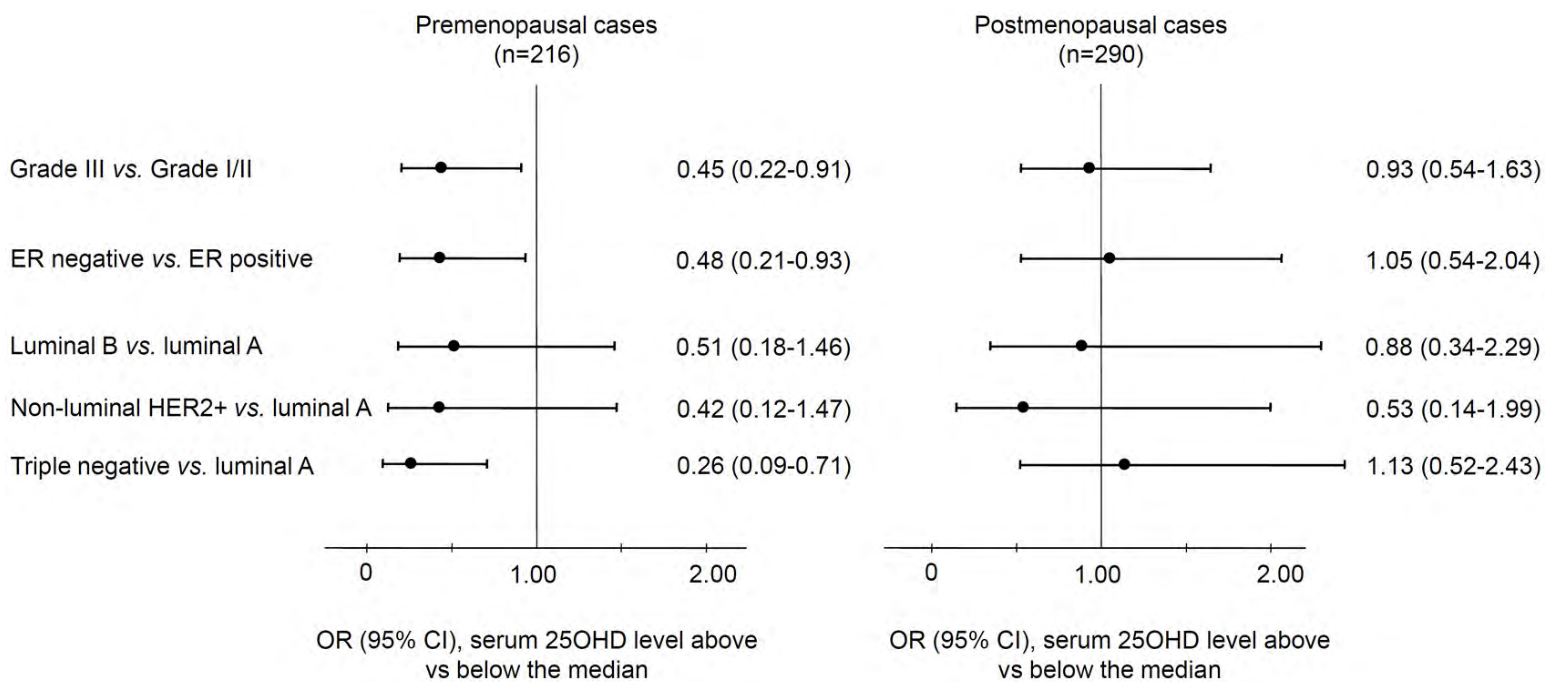
Footnote: Season-standardized serum 25-hydroxyvitamin D (25OHD) concentrations were stratified into above and below the median in healthy controls by menopausal status. Odds ratios (OR) and 95% confidence intervals (CI) were derived from multinomial logistic regression, adjusted for age at diagnosis and BMI. Further adjustment for physical activity did not significantly change the results (data not shown).

Figure 1.2. Case-control analysis of breast cancer risk by prognostic characteristics with an incremental increase of 10 ng/mL serum 25-hydroxyvitamin D levels in premenopausal and postmenopausal women



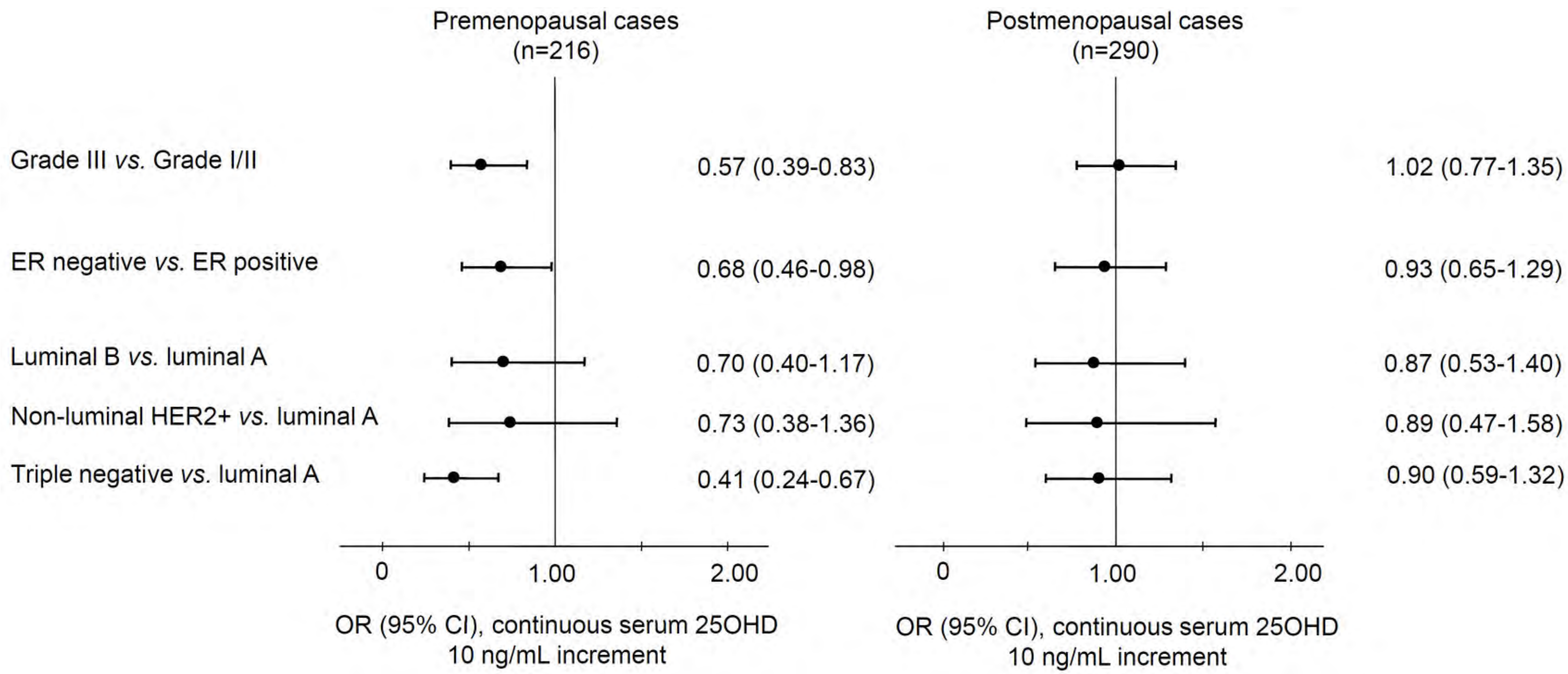
Footnote: Season-standardized serum 25-hydroxyvitamin D (25OHD) concentrations were entered into the regression models as a continuous variable. Odds ratios (OR) and 95% confidence intervals (CI) of an incremental increase of 10 ng/mL 25OHD were derived from multinomial logistic regression, adjusted for age at diagnosis and BMI. Further adjustment for physical activity did not significantly change the results (data not shown).

Figure 1.3. Case-only analysis of prognostic characteristics with serum 25-hydroxyvitamin D levels in premenopausal and postmenopausal women diagnosed with invasive cancer



Footnote: Season-standardized serum 25-hydroxyvitamin D (25OHD) concentrations were stratified into above and below the median levels in healthy controls by menopausal status. Odds ratios (OR) and 95% confidence intervals (CI) were derived from logistic regression (histologic grade and ER status) or multinomial logistic regression (molecular subtype), adjusted for age at diagnosis and BMI. Further adjustment for physical activity did not significantly change the results (data not shown).

Figure 1.4. Case-only analysis of prognostic characteristics with an incremental increase of 10 ng/mL serum 25-hydroxyvitamin D levels in premenopausal and postmenopausal women diagnosed with invasive cancer



Footnote: Season-adjusted serum 25-hydroxyvitamin D (25OHD) concentrations were entered into the regression models as a continuous variable. Odds ratios (OR) and 95% confidence intervals (CI) of an incremental increase of 10 ng/mL 25OHD were derived from logistic regression (histological grade and ER status) or multinomial logistic regression (molecular subtype), adjusted for age at diagnosis and BMI. Further adjustment for physical activity did not significantly change the results (data not shown).

Table 2.1. Descriptive characteristics of African American and European American women by case-control status in the Women's Circle of Health Study (WCHS)

Characteristics	**African American**			**European American**		
	Case (n=547)	**Control (n=461)**	**P**	**Case (n=381)**	**Control (n=382)**	**P**
	Mean (SD)	Mean (SD)		Mean (SD)	Mean (SD)	
Age	51.7 (10.0)	49.8 (9.9)	0.003	51.0 (8.4)	50.9 (8.3)	0.82
Body mass index	31.2 (6.7)	31.6 (7.8)	0.48	26.8 (5.8)	27.7 (7.1)	0.06
% European ancestry	0.09 (0.15)	0.10 (0.16)	0.19	0.98 (0.07)	0.99 (0.03)	0.07
	Count (%)	Count (%)		Count (%)	Count (%)	
Menopausal status			0.14			0.17
Premenopausal	337 (61.6)	263 (57.0)		235 (61.7)	217 (56.8)	
Postmenopausal	210 (38.4)	198 (43.0)		146 (38.3)	165 (43.2)	
Family history			0.13			0.001
Yes	82 (15.0)	54 (11.7)		104 (27.3)	67 (17.5)	
No	465 (85.0)	407 (88.3)		277 (72.7)	315 (82.5)	
Education			0.06			<0.001
Less than high school	76 (13.9)	55 (11.9)		9 (2.4)	4 (1.1)	
High school	175 (32.0)	122 (26.5)		80 (21.0)	44 (11.5)	
College and above	296 (54.1)	284 (61.6)		292 (76.6)	334 (87.4)	
Hormone replacement therapy			0.74			0.47
Yes	79 (14.5)	62 (14.2)		96 (25.3)	88 (23.0)	
No	464 (85.5)	397 (85.8)		284 (74.7)	294 (77.0)	

Footnote: For continuous variables, p-values were based on t-test; for categorical variable, *p*-values were based on Chi-square test. The counts of some variables did not add up to the total due to occasional missing values.

Table 2.2. SNPs in *VDR* and *CYP24A1* and differential associations with breast cancer risk between African American and European American women in WCHS

Gene	SNP	Genotype	African American			European American			$P_{interaction}$
			# case /control	Adjusted OR (95% CI)	P_{trend}	# case /control	Adjusted OR (95% CI)	P_{trend}	
VDR	rs11608702	AA	330/261	1.00	0.37	166/190	1.00	0.02	0.01
		AT	175/175	0.80 (0.61-1.04)		160/159	1.15 (0.84-1.58)		
		TT	37/25	1.13 (0.65-1.95)		55/31	1.88 (1.14-3.09)		
VDR	rs12721364	GG	520/420	1.00	0.01	303/298	1.00	0.90	0.02
		GA/AA	24/40	0.53 (0.31-0.90)		78/82	0.98 (0.68-1.41)		
VDR	rs2239186	AA	497/393	1.00	0.001	265/256	1.00	0.46	0.01
		AG/GG	47/68	0.53 (0.35-0.79)		115/125	0.85 (0.62-1.17)		
VDR	rs11568820 (Cdx2)	GG	18/26	1.00	0.04	234/232	1.00	0.68	0.04
		GA	143/140	1.55 (0.79-3.03)		129/132	0.99 (0.72-1.36)		
		AA	384/295	1.94 (1.01-3.74)		18/18	0.83 (0.39-1.75)		
CYP24A1	rs912505	AA	173/139	1.00	0.71	236/216	1.00	0.02	0.05
		AG	268/244	0.89 (0.66-1.19)		132/139	0.80 (0.59-1.10)		
		GG	104/77	1.14 (0.78-1.66)		13/27	0.36 (0.17-0.76)		
CYP24A1	rs3787555	CC	379/331	1.00	0.14	206/183	1.00	0.03	0.02
		CA	154/123	1.17 (0.88-1.56)		149/155	0.81 (0.59-1.10)		
		AA	14/7	1.88 (0.73-4.83)		25/41	0.50 (0.28-0.89)		

Footnote: Odds ratio (OR) and 95% confidence interval (CI) are adjusted for covariates including age, proportion of European ancestry, body mass index, family history of breast cancer, and education. P_{trend} was for genetic dose-response by coding genotypes as 0, 1 and 2 based on the number of variant allele. $P_{interaction}$ was for the differences in odds ratios between African American and European American women, and $P_{interaction} < 0.10$ was deemed significant.

Table 2.3. SNPs in *VDR* and *CYP24A1* and differential association with ER specific breast cancer risk among African American and European American women

Gene	SNP	Genotype	African American			European American			$P_{interaction}$
			# case/control	OR (95% CI)	P_{trend}	# case/control	OR (95% CI)	P_{trend}	
Estrogen receptor positive breast cancer									
VDR	rs10783218	GG	178/304	1.00	0.82	194/352	1.00	0.04	0.04
		GA/AA	85/150	0.96 (0.69-1.34)		20/17	2.05 (1.02-4.12)		
Estrogen receptor negative breast cancer									
VDR	rs3819545	AA	71/242	1.00	0.04	22/162	1.00	0.38	0.04
		AG	45/168	0.91 (0.59-1.40)		26/156	1.22 (0.66-2.26)		
		GG	3/43	0.23 (0.07-0.77)		11/52	1.40 (0.62-3.15)		
CYP24A1	rs927650	GG	66/258	1.00	0.62	10/116	1.00	0.003	0.10
		GA	47/170	1.09 (0.71-1.68)		29/186	1.76 (0.81-3.78)		
		AA	8/27	1.18 (0.50-2.79)		20/68	3.46 (1.50-7.96)		
CYP24A1	rs1570669	GG	15/81	1.00	0.69	32/158	1.00	0.03	0.05
		GA	69/219	1.77 (0.94-3.31)		24/162	0.71 (0.40-1.27)		
		AA	37/154	1.36 (0.69-2.67)		3/50	0.28 (0.08-0.97)		
CYP24A1	rs2209314	AA	102/393	1.00	0.33	46/208	1.00	0.004	0.003
		AG/GG	19/61	1.34 (0.74-2.40)		13/162	0.38 (0.20-0.73)		
CYP24A1	rs3787555	CC	79/326	1.00	0.02	25/175	1.00	0.91	0.09
		CA	37/122	1.42 (0.90-2.24)		29/155	1.25 (0.69-2.24)		
		AA	5/7	3.79 (1.11-12.91)		5/38	0.83 (0.30-2.35)		
CYP24A1	rs2762941	AA	16/120	1.00	0.004	24/132	1.00	0.54	0.05
		AG	61/212	1.97 (1.07-3.61)		26/168	0.89 (0.49-1.65)		
		GG	44/122	2.62 (1.38-4.98)		9/68	0.78 (0.34-1.78)		
CYP24A1	rs4809959	GG	32/133	1.00	0.99	14/131	1.00	0.01	0.07
		GA	64/223	1.13 (0.70-1.83)		26/172	1.43 (0.71-2.88)		
		AA	25/98	0.98 (0.54-1.78)		19/66	2.71 (1.25-5.86)		
CYP24A1	rs2585428	GG	27/109	1.00	0.65	26/90	1.00	0.006	0.02
		GA	66/246	1.12 (0.67-1.87)		22/175	0.46 (0.24-0.87)		
		AA	28/100	1.15 (0.63-2.10)		11/105	0.36 (0.17-0.79)		

Footnote: Odds ratio (OR) and 95% confidence interval (CI) are adjusted for covariates including age, proportion of European ancestry, body mass index, family history of breast cancer, and education. P_{trend} was for genetic dose-response by coding genotypes as 0, 1 and 2 based on the number of variant allele. $P_{interaction}$ was for the differences in odds ratios between African American and European American women, and $P_{interaction} < 0.10$ was deemed significant.

Table 2.4. Changes in risk of estrogen receptor (ER)-negative breast cancer by race with inclusion of SNPs in *CYP24A1*

Model	Variable	Adjusted OR (95% CI)	P
Base model	race (AA vs. EA)	1.53 (1.06-2.22)	0.02
Base model + SNPs	race (AA vs. EA)	1.20 (0.80-1.79)	0.38
	rs2209314 (AG/GG vs. AA)	0.57 (0.36-0.89)	0.01
	rs2762941 (AG vs. AA)	1.47 (0.96-2.25)	0.04
	rs2762941 (GG vs. AA)	1.88 (1.15-3.06)	

Footnote: Covariates included in the base model were age at diagnosis, body mass index, family history of breast cancer, education, and race. Odds ratio (OR) and 95% confidence interval (CI) for race after adjustment for other covariates are shown. Based on this model, 7 SNPs in *CYP24A1* (rs927650, rs1570669, rs2209314, rs3787555, rs2762941, rs4809959, and rs2585428) and one SNP in *VDR* (rs3819545) that were associated with ER-negative breast cancer risk in either African American (AA) or European American (EA) women were entered and backward selected. Two SNPs, rs2209314 and rs2762941, remained in the final model with a $p<0.05$. ORs and 95% CIs for race and those two SNPs were shown.

Figure 2.1. Proportion of frank vitamin D deficiency (<10 ng/ml) in African American and European American healthy women

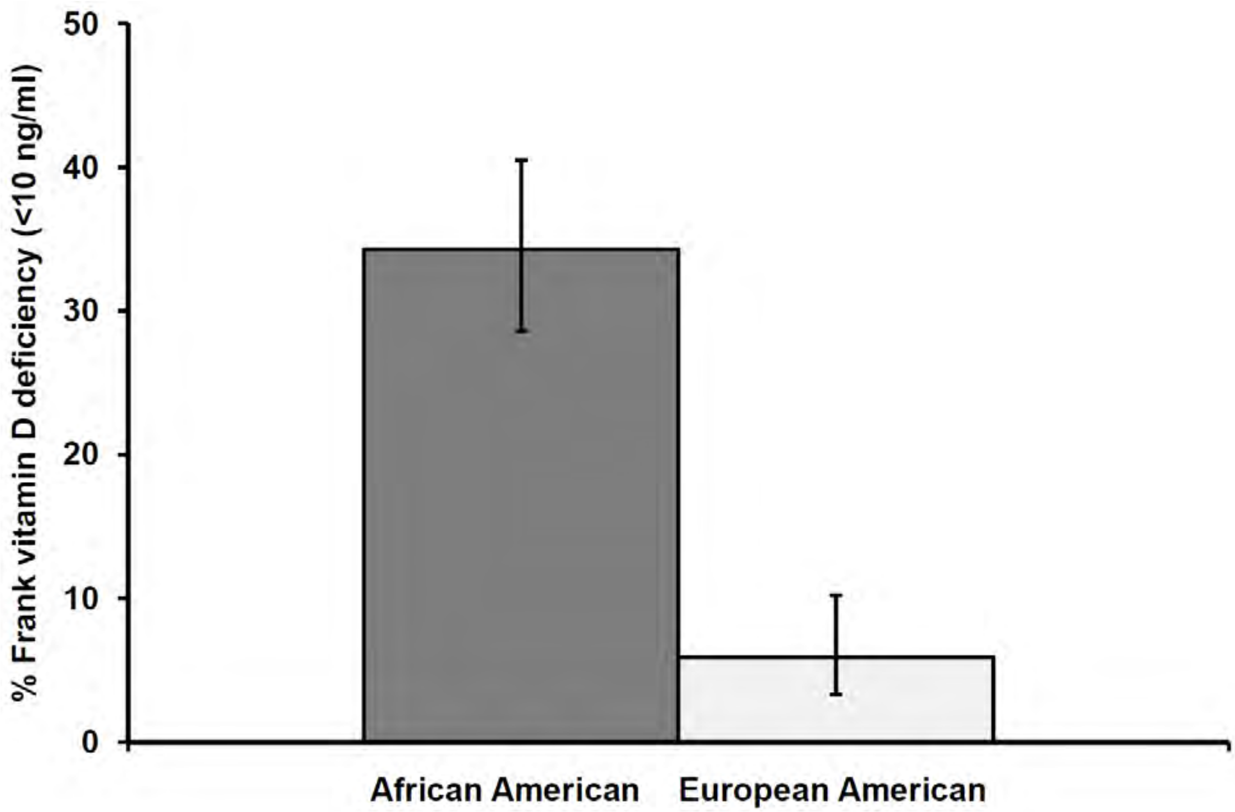


Figure 2.2 Plot of univariate analysis *p*-values for associations with breast cancer risk and linkage disequilibrium of vitamin D receptor (*VDR*) SNPs in African American and European American women

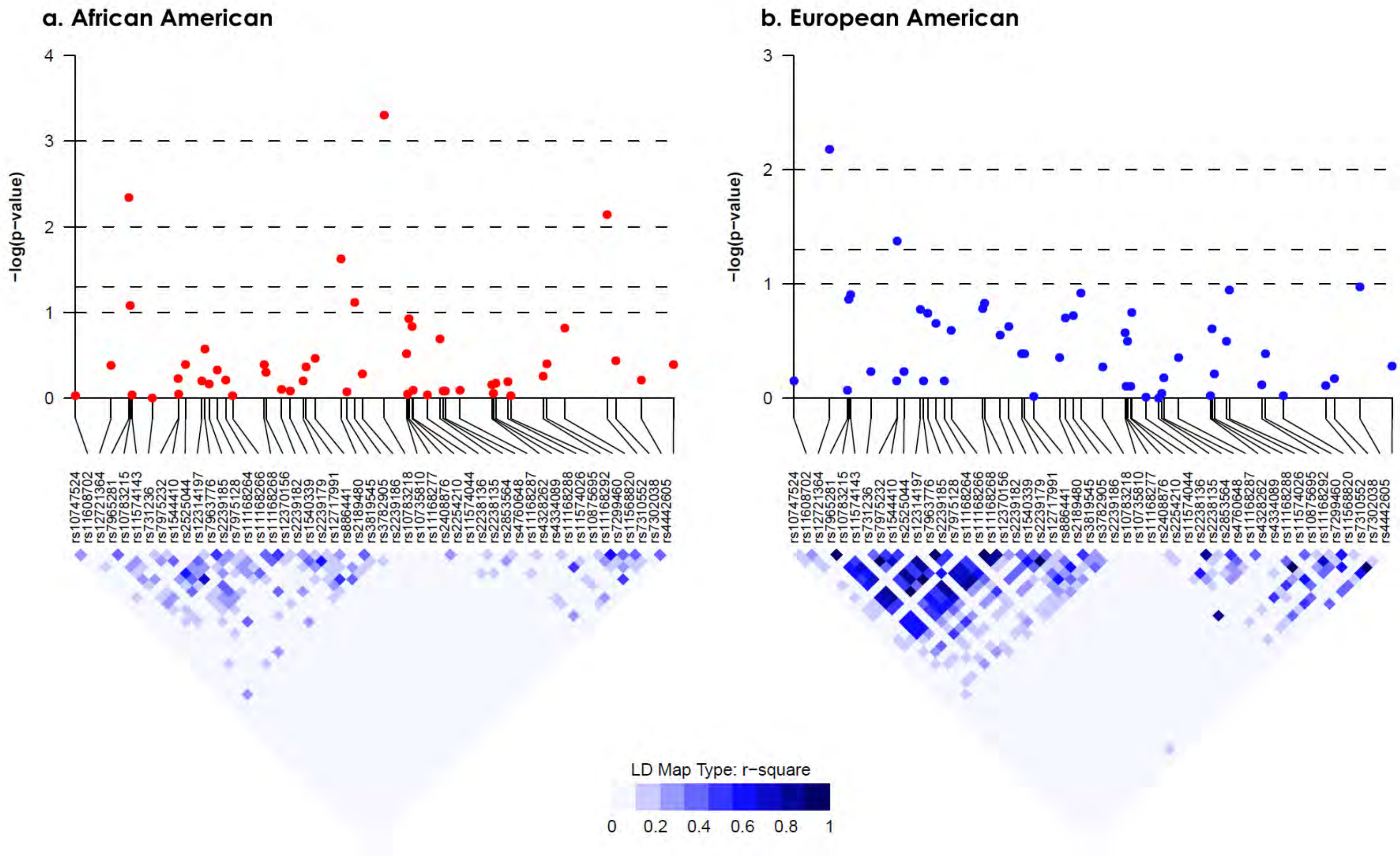
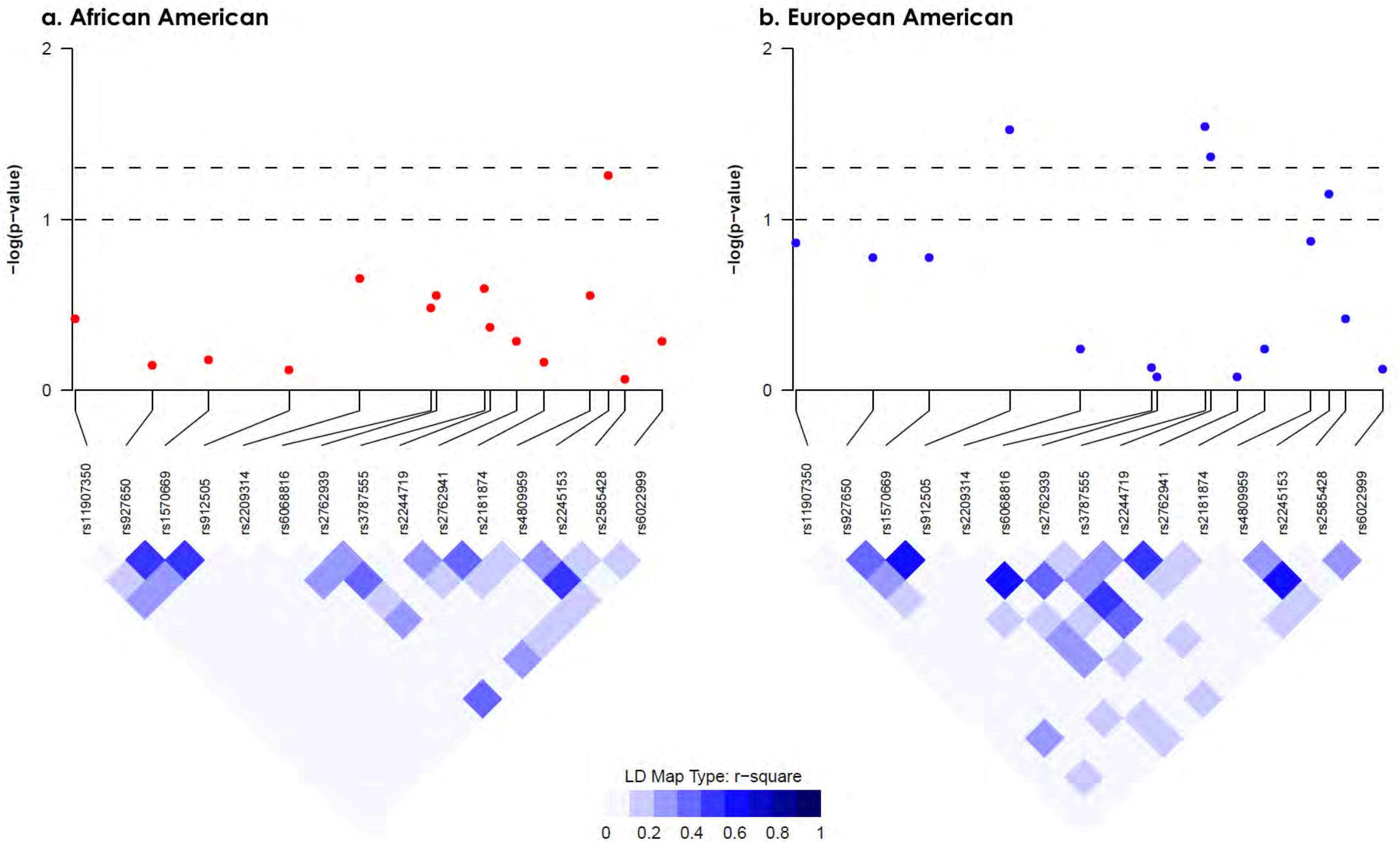


Figure 2.3. Plot of univariate analysis *p*-values for associations with breast cancer risk and linkage disequilibrium of 24-hydroxylase (*CYP24A1*) SNPs in African American and European American women



APPENDICES

1. An abstract submitted to AACR 2011 annual meeting
2. An abstract submitted to DOD BCRP 2011 meeting
3. Published paper on PLoS One.

Common genetic variations in *VDR*, *CYP27B1* and *CYP24A1* genes and breast cancer risk in African American and European American women in the Women's Circle of Health Study

Song Yao, Gary Zirpoli, Chi-Chen Hong, Li Tang, Hua Zhao, Lara Sucheston, Jyoti Shankar, Michelle Roberts, Melanie Ruszczyk, Gregory Ciupak, Warren Davis, Helena Hwang, Susan McCann, Elisa Bandera, Christine Ambrosone

Common single nucleotide polymorphisms (SNPs) in vitamin D receptor (*VDR*) have been examined with breast cancer risk in populations of European ancestry. Because of the striking differences in vitamin D levels and in SNP linkage disequilibrium (LD) between African Americans (AA) and European Americans (EA), it is hypothesized that associations of *VDR* SNPs with breast cancer risk may differ by race.

A total of 67 multi-population tagSNPs in the *VDR*, *CYP27B1* and *CYP24A1* genes were genotyped in 928 breast cancer cases (547 AA and 381 EA) and 843 controls (461 AA and 382 EA) recruited through the Women's Circle of Health Study (WCHS), a case-control study based in New York City and seven counties in New Jersey. Cochran-Armitage test for trend was used for single SNP analysis, and LD block-based haplotype analysis was performed, with multiple comparisons corrected by permutation. In addition, 25-hydroxyvitamin D (25OHD) levels were measured in the banked serum samples from 242 AA and 187 EA healthy women.

Four *VDR* SNPs were associated with breast cancer risk in AA women, and rs2239186 remained significant (p=0.03) after multiple comparison correction. Women with the AG/GG genotype of rs2239186 had a lower risk of breast cancer (OR=0.53, 95% CI=0.35-0.79) than those with the AA genotype, consistent with a three-SNP haplotype carrying the G allele of rs2239186 (OR=0.55, 95% CI=0.38-0.81) in AA women. In EA women, 2 SNPs in the *VDR* gene and 3 SNPs in the *CYP24A1* gene were associated with breast cancer risk. EA women with the TT genotype of the *VDR* SNP rs11608702 had a significantly increased risk (OR=1.88, 95% CI=1.14-3.09), which were confirmed in an analysis of a two-SNP haplotype carrying the T allele (OR=1.44, 95% CI=1.12-1.85). In addition, a four-SNP haplotype in the *CYP24A1* gene was associated with reduced cancer risk in EA women (OR=0.67, 95% CI=0.51-0.88). When stratified by menopausal status, the association of rs7975232 in *VDR* in EA women was stronger in postmenopausal women (OR=2.24, 95% CI=1.19-4.21); and the association of another *VDR* SNP rs886441 in AA women was stronger in premenopausal women (OR=2.27, 95% CI=1.32-3.90). Lastly, we found significant differences in 25OHD levels between AA and EA women; the least square mean and standard error were 14.9±0.5 vs 21.4±0.6 ng/mL (p<0.0001), respectively, after adjusting for age, BMI and season of blood collection. Dietary intake of vitamin D helped explain only 3% of the circulating levels.

In conclusion, we found different SNPs associated with breast cancer risk between AA and EA women. Our findings support the hypothesis that the role of vitamin D-related genetic variations may be race-specific, possibly due to differences in linkage disequilibrium structure and in endogenous vitamin D levels between the two groups.

Vitamin D and breast cancer in African American and European American women

Song Yao, Gary Zirpoli, Chi-Chen Hong, Li Tang, Hua Zhao, Lara Sucheston, Jyoti Shankar, Michelle Roberts, Melanie Ruszczyk, Gregory Ciupak, Warren Davis, Helena Hwang, Susan McCann, Elisa Bandera, Christine Ambrosone

BACKGROUN AND OBJECTIVES

Evidence from laboratory and epidemiologic studies suggests that vitamin D may be associated with breast cancer (BC) of poor prognosis, particularly the triple-negative (TN) subtype. If this holds true, it may help explain the strikingly high incidence of (TNBC) among young African American (AA) women, because AAs have significantly lower vitamin D levels than European Americans (EAs). To test these hypotheses, we proposed two objectives: 1) to examine associations between serum levels of 25-hydroxyvitamin D (25OHD) and BC prognostic characteristics defined by tumor grade, ER status, and TN status; 2) to examine predicted vitamin D levels and single nucleotide polymorphisms (SNPs) in vitamin D receptor (*VDR*) and metabolizing genes with BC risk and tumor characteristics in AAs and EAs.

METHODS

Pre-treatment serum samples for 25OHD assays were available from 579 EA BC cases and 574 EA controls. Levels of 25OHD were compared by tumor characteristics, with control for age, BMI and season of blood collection. Odds ratios (OR) and 95% confidence intervals (CIs) were estimated by logistic regression. In a second population of 547 AA and 381 EA BC patients and 461 AA and 382 EA controls, 68 multi-population tagSNPs in *VDR*, *CYP27B1* and *CYP24A1* genes were typed together with 110 ancestry informative markers. Genotype analyses were performed with PLINK and multiple comparisons were adjusted by permutation. Because serum was only available from a subset of women in the second population, predict vitamin D scores based on a linear regression model with factors influencing vitamin D levels, as well as SNPs associated with skin pigmentation, will be computed and examined with risk of BC in AA and EA women.

RESULTS

BC cases had lower 25OHD than controls (22.8 vs 26.2 ng/mL, $p<0.001$). Among premenopausal women, serum 25OHD were lower in those with high- vs low-grade tumors, and ER- vs ER+ tumors ($p\leq0.03$), and were lowest among TNBC cases (17.5 ng/mL). Every 10 ng/mL increase in 25OHD was associated with a 64% lower odds of having TNBC (OR=0.36, 95% CI=0.22-0.56).

In AAs 4 *VDR* SNPs were associated with BC risk, including rs2239186, which remained significant after control for multiple comparison (p =0.03). Women with GG/GA had lower risk of BC than those with AA genotype (OR=0.53, 95% CI=0.35-0.79). A three-SNP haplotype with the G allele was related to reduced risk (OR=0.55, 95% CI=0.38-0.81). In EA women, however, 5 other SNPs were in association with BC risk, including rs11608702 in *VDR* associated with increased risk (OR=1.88, 95% CI=1.14-3.09). Interaction testing confirmed differential associations of the above SNPs between AA and EA women. The work of predicted vitamin D scores and breast cancer risk in AAs and EAs is currently undergoing.

CONCLUSIONS

We found lower vitamin D levels in association with BC of poor prognosis, particularly TNBC among premenopausal women. A number of SNPs in *VDR* and *CYP24A1* genes associated with BC risk in AA and EA women; nevertheless, the relationships differed between the two groups, suggesting race-specific effects, which may be related to cancer disparity. Our findings indicate that, vitamin D may play a role in reducing risk of BC associated with poor prognosis, particularly TNBC. Because TNBC is over-represented in AA women before menopause, this racial disparity may be reduced by maintaining sufficient vitamin D levels, which can be readily achieved by supplementation and sun exposure.

PLoS one

Pretreatment Serum Concentrations of 25-Hydroxyvitamin D and Breast Cancer Prognostic Characteristics: A Case-Control and a Case-Series Study

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Abstract

Background: Results from epidemiologic studies on the relationship between vitamin D and breast cancer risk are inconclusive. It is possible that vitamin D may be effective in reducing risk only of specific subtypes due to disease heterogeneity.

Methods and Findings: In case-control and case-series analyses, we examined serum concentrations of 25-hydroxyvitamin D (25OHD) in relation to breast cancer prognostic characteristics, including histologic grade, estrogen receptor (ER), and molecular subtypes defined by ER, progesterone receptor (PR) and HER2, among 579 women with incident breast cancer and 574 controls matched on age and time of blood draw enrolled in the Roswell Park Cancer Institute from 2003 to 2008. We found that breast cancer cases had significantly lower 25OHD concentrations than controls (adjusted mean, 22.8 versus 26.2 ng/mL, $p<0.001$). Among premenopausal women, 25OHD concentrations were lower in those with high- versus low-grade tumors, and ER negative versus ER positive tumors ($p\leq0.03$). Levels were lowest among women with triple-negative cancer (17.5 ng/mL), significantly different from those with luminal A cancer (24.5 ng/mL, $p=0.002$). In case-control analyses, premenopausal women with 25OHD concentrations above the median had significantly lower odds of having triple-negative cancer (OR = 0.21, 95% CI = 0.08–0.53) than those with levels below the median; and every 10 ng/mL increase in serum 25OHD concentrations was associated with a 64% lower odds of having triple-negative cancer (OR = 0.36, 95% CI = 0.22–0.56). The differential associations by tumor subtypes among premenopausal women were confirmed in case-series analyses.

Conclusion: In our analyses, higher serum levels of 25OHD were associated with reduced risk of breast cancer, with associations strongest for high grade, ER negative or triple negative cancers in premenopausal women. With further confirmation in large prospective studies, these findings could warrant vitamin D supplementation for reducing breast cancer risk, particularly those with poor prognostic characteristics among premenopausal women.

Citation: Yao S, Sucheston LE, Millen AE, Johnson CS, Trump DL, et al. (2011) Pretreatment Serum Concentrations of 25-Hydroxyvitamin D and Breast Cancer Prognostic Characteristics: A Case-Control and a Case-Series Study. PLoS ONE 6(2): e17251. doi:10.1371/journal.pone.0017251

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Introduction

Vitamin D is a secosteroid hormone critical to bone health and other biological pathways [1]. Circulating 25-hydroxyvitamin D (25OHD), the widely-used biomarker for endogenous levels of vitamin D, as well as proxies of vitamin D exposure, such as sun exposure and dietary and supplementary intake, have been evaluated in relation to risk of various malignancies [2]. However, consistent associations have only been demonstrated for colorectal cancer [3,4]. Despite numerous experimental studies repeatedly showing anti-neoplastic activities of vitamin D on breast cancer [5,6], findings from epidemiologic studies and randomized trials are not definitive [7,8,9].

It is possible that tumor heterogeneity in breast cancer may mask associations. Clinical markers including estrogen receptor (ER), progesterone receptor (PR) and tumor grade have long been used to classify breast cancer subtypes associated with differential prognosis and response to cancer therapy. These crude subtypes

were refined by recent gene expression microarray studies, which clustered breast tumors into five major molecular subtypes [10,11]. A validated panel of immunohistochemical (IHC) markers have been developed to approximate the classification of these subtypes, including luminal A (ER+ and/or PR+ and HER2-), luminal B (ER+ and/or PR+ and either HER2+ or Ki-67+), non-luminal HER2+ (ER-, PR-, and HER2+), basal-like (ER-, PR-, HER2-, CK5/6+ and/or HER1+), and unclassified (ER-, PR-, HER2-, CK5/6-, and HER1-) [12,13,14]. Several studies have shown that reproductive risk factors differ for the particular molecular subtypes [15,16,17]; and it is likely that relationships between vitamin D and breast cancer risk may also vary according to subtypes. Interestingly, in the Physicians' Health Study, blood levels of 1,25-dihydroxyvitamin D were strongly associated with the risk of aggressive, but not total prostate cancer [18]. Similar differential associations may also exist for breast cancer.

The definition of breast tumors that are 'triple negative', i.e., lack of expression of ER, PR and HER2, largely overlap with that of basal-like tumors and is sometimes used as a proxy for the latter. Basal-like or triple negative tumors pose a major challenge for breast cancer treatment, because it does not repond to hormonal therapy targeting ER or trastuzumab targeting HER2. In a recent case-series study, women with triple negative breast cancer had the lowest serum 25OHD concentrations compared to those with other molecular cancer subtypes [19]. However, only 15 patients with triple negative cancer were included in that analysis. In a case-controls study of 579 women with primary incident breast cancer and 574 controls matched on age and time of blood collection, we examined serum concentrations of 25OHD at diagnosis or enrollment, with a particular focus on associations with breast cancer prognostic characteristics, specifically, tumor histologic grade, ER status, and molecular subtypes characterized by ER, PR and HER2.

Methods

Study population

Data and specimens from women with breast cancer and healthy controls were obtained from the Data Bank and Biorepository (DBBR) at Roswell Park Cancer Institute (RPCI). The DBBR, as previously described [20], is a comprehensive data and sample bank containing pretreatment biospecimens that are rigorously collected and processed, with comprehensive clinical and epidemiologic data. Briefly, patients newly diagnosed with cancer at RPCI are invited to participate during their initial visit with the surgical oncologist. After consent, blood samples are collected (prior to any treatment, including surgery, for breast cancer) in phlebotomy when specimens for clinical measures are drawn, transported to the laboratory through a pneumatic tube system, and processed within one hour of blood draw. Specimens are maintained in liquid nitrogen until analysis. The average time interval between the time of diagnosis and the time of blood draw for the women in our study was 27 days.

Inclusion criteria for breast cancer cases in the study were: self-identified as non-Hispanic white, histologically confirmed primary, incident, female breast cancer, and no prior cancer history except non-melanoma skin cancer. Healthy controls were identified from family members and friends of the patients and other visitors to RPCI or from volunteers recruited from community events, and blood was drawn and processed at RPCI in the same manner as the cases. For this study, controls were matched to cases on five year age category and month of blood collection. Those who were family members or friends of the breast cancer cases were not included in the study. Self-administered questionnaires were used to collect data on demographics, reproduction, medical history, family histories of cancer, and lifestyle factors including physical activity. Self-reported physical activity compared to same age peers was used as an estimate for sun exposure. In addition, questionnaire data on activities including walking, running, cycling, and golfing were included as alternative estimates for sun exposure. A food frequency questionnaire was administered, and questions on supplement use were included. Ninety-two percent (92%) of the women in this study had questionnaires returned. Postmenopausal status in the study was defined as women who experienced 12 consecutive months of amenorrhea, or women who underwent bilateral salpingo-oophorectomy. This study was approved by the Institutional Review Board at RPCI.

Clinical data and breast cancer prognostic characteristics

Patients' clinical data, including tumor stage, histologic grade and ER, PR and HER2 status, were obtained from a clinical database maintained by the RPCI breast program, and supplemented with data from abstracted medical records and the RPCI Tumor Registry. Because IHC of CK 5/6 or EGFR was not routinely performed in pathology, we instead defined four molecular subtypes in our study based on ER, PR and HER2 as follows: luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), non-luminal HER2+ (ER-, PR- and HER2+), and triple negative (ER-, PR- and HER2-). As such, we were not able to distinguish the basal-like and unclassified subtypes, both of which were included in the triple negative group in our study. However, it has been shown that the prognostic significance of the triple negative subtype is similar to that of the basal-like subtype [21]. ER, PR and HER2 status were measured by IHC in pathology, and amplification of *HER2* gene was tested by fluorescence *in situ* hybridization (FISH) when IHC scored 2+. Histologic grade, ER status and molecular subtypes defined by ER, PR and HER2 were used as three independent prognostic characteristics for breast cancer. In addition, tumor stage was also included for analysis.

Serum 25-hydroxyvitamin D assay

Considering the potential variability of 25OHD assays, we first tested assay performance on pilot samples in two different laboratories both running the immunochemiluminometric assay on the DiaSorin Liasion automated instrument. At one laboratory, the coefficient of variation (CV) was 19%, which was considered inappropriate for the purpose of this study. At another laboratory (Heartland Assay, Ames, IA), the CV was 6.5%, and this laboratory was chosen. For the entire batch of samples analyzed for cases and controls, the CV was 8.8%.

Statistical analysis

For univariate analysis, we first compared serum 25OHD concentrations in the healthy controls by a number of selected factors that might affect vitamin D levels, using non-parametric tests. To compare serum 25OHD concentrations by case-control status or by tumor prognostic characteristics, we used a generalized linear model controlling for age, body mass index (BMI) and season of blood collection, which had independent effects on serum 25OHD concentrations ($p<0.05$). Least square means and standard errors of 25OHD concentrations were derived separately for each of the tumor characteristics. Physical activity was not associated with serum 25OHD levels after control for BMI, and adding it to the models had little impact on the results. Thus, results without additional adjustment for physical activity are presented.

To examine serum 25OHD levels in relation to breast cancer prognostic characteristics, we performed two types of analyses, including case-control analysis, where healthy controls were used as a referent group, and case-series analysis, where women with better prognostic characteristics (grade I/II, ER+, or luminal A subtype) were used as a referent group and women with carcinoma *in situ* (CIS) were excluded. Logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI) associated with 25OHD levels. When outcomes had more than two levels, multinomial logistic regression models were fitted. Considering large seasonal variations of 25OHD concentrations due to change of solar ultra-violet B intensity in the Northeastern United States through a year, we computed season-standardized 25OHD concentrations by locally weighted multinomial regression to determine the cut-off points of vitamin D levels for logistic regression, following the approach described by Ahn and colleagues [22].

For case-control analysis, season-standardized vitamin D levels were defined as follows: deficient (<20.0 ng/mL), insufficient (20.0–29.9 ng/mL), and sufficient (≥30.0 ng/mL). For case-series analysis of prognostic characteristics, because the number of cases was limited in some categories, we dichotomized season-standardized 25OHD concentrations based on the medians in healthy controls. In addition, we also treated season-standardized 25OHD concentrations as a continuous variable in the regression models and computed the ORs and 95% CIs associated with an incremental increase of 10 ng/mL of 25OHD. Because etiologic pathways of breast cancer may differ between premenopausal and postmenopausal women, we first performed analyses for all women, and then stratified the analyses by menopausal status. All analyses were performed using SAS 9.2 with two-sided significance level of 0.05 (SAS Institute, Cary, NC).

Results

Table 1 shows serum 25OHD concentrations according to selected demographic and lifestyle characteristics of the control population. Younger women tended to have higher 25OHD levels than older women, although the differences were not statistically significant. There were apparent seasonal variations of serum 25OHD concentrations, with a peak during summer season. Circulating 25OHD concentrations were inversely associated with BMI, and positively associated with physical activity. Women who had higher dietary vitamin D intake or took vitamin D supplements had higher circulating concentrations.

The median of serum 25OHD concentrations in breast cancer cases and controls were 22.8 ng/mL and 26.2 ng/mL, respec-

Table 1. Serum 25-hydroxyvitamin D concentrations by demographic and lifestyle characteristics among healthy controls.

Characteristics	N (%)[1]	Serum 25OHD, median (IQR), ng/ml	P-value[2]
Age, year			0.56
<50	202 (35.2)	28.3 (19.8–36.3)	
50–59	169 (29.4)	27.0 (19.2–33.4)	
60–69	127 (22.1)	26.8 (19.4–32.3)	
≥70	76 (13.2)	26.7 (19.8–33.5)	
Season of blood collection			<0.001
Spring (Mar–May)	99 (17.2)	25.7 (15.9–33.2)	
Summer (Jun–Aug)	175 (30.5)	30.5 (22.9–36.9)	
Fall (Sep–Nov)	135 (23.5)	25.2 (19.5–32.8)	
Winter (Dec–Feb)	165 (28.7)	24.7 (16.5–31.8)	
BMI, kg/m^2			<0.001
<25.0	184 (33.0)	30.7 (24.4–38.8)	
25.0–29.9	198 (35.5)	27.5 (20.7–33.2)	
≥30.0	175 (31.4)	21.6 (15.4–28.2)	
Physical activity			<0.001
More active	264 (46.2)	29.3 (22.2–36.5)	
Normal	198 (34.5)	24.3 (18.8–31.9)	
Less active	110 (19.2)	25.1 (16.1–33.2)	
Dietary vitamin D			0.003
Q1 (<42 IU/day)	146 (25.4)	24.0 (17.8–32.2)	
Q2 (42–147 IU/day)	134 (23.3)	27.3 (20.6–36.8)	
Q3 (148–329 IU/day)	142 (24.7)	27.6 (20.6–32.8)	
Q4 (≥330 IU/day)	152 (26.5)	27.3 (21.0–37.6)	
Supplementary vitamin D			<0.001
Yes	259 (45.1)	28.1 (22.2–35.0)	
No	315 (54.9)	24.9 (16.6–33.0)	

[1]For some characteristics, the numbers did not add up to the totals due to missing data.
[2]P-values were derived from Wilcoxon rank test for variables with two levels and Kruskal-Wallis test for variables with more than two levels. Abbreviation: IQR, interquartile range.
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tively. After control for seasonal variations, a majority of the controls were either vitamin D deficient (25.8%) or insufficient (35.7%), and only 38.5% of them had a sufficient level of 30 ng/mL or higher. The proportion of vitamin D deficiency was even higher in cases (38.5%), and only a small proportion of them were considered vitamin D sufficient (21.4%) ($p<0.001$). As shown in Table 2, compared to women who were vitamin D deficient, those with sufficient levels had a 63% reduction in odds of breast cancer (OR = 0.37, 95% CI = 0.27–0.51). Every 10 ng/mL incremental increase of 25OHD concentrations was associated with an estimated reduction of breast cancer odds by one third (OR = 0.67, 95% CI = 0.59–0.75), which was significant in both premenopausal and postmenopausal women.

When pre- and postmenopausal women with invasive breast cancer were considered together, there were no significant differences in serum 25OHD concentrations by histologic grade or ER status (data not shown). However, women with triple negative breast cancer had the lowest vitamin D concentrations among the 4 molecular subtypes after control for age, BMI and season of blood collection (least square mean ± standard error: 23.0±0.5, 21.3±1.3, 21.6±1.6 and 19.9±1.1 ng/mL for luminal A, luminal B, non-luminal HER2+ and triple negative subtypes, respectively, $p = 0.046$). In addition, there was an inverse relationship between serum 25OHD concentrations and tumor stage (26.5±1.0, 23.2±0.5, 21.3±0.7 and 21.9±2.0 ng/mL for stage 0 [CIS], stage I, stage II/IIIA, and stage IIIB/IIIC/IV, respectively, $p<0.001$).

When stratifying by menopausal status, serum 25OHD levels did not differ by tumor characteristics among postmenopausal women, but there were notable differences among premenopausal women (Table 3). Those diagnosed with invasive breast cancer, especially late stage cancer, had significantly lower 25OHD concentrations than those with CIS ($p<0.001$). Among premenopausal women with invasive breast cancer, those who had high grade or ER negative cancer had lower serum 25OHD concentrations than those with high grade or ER positive cancer ($p\leq0.03$). Moreover, premenopausal women diagnosed with triple negative cancer tumors had the lowest concentrations compared to those with the other three molecular subtypes ($p = 0.002$).

In case-control analyses, ORs and 95% CIs of breast cancer by menopausal status and tumor prognostic characteristics are plotted in Figure 1. Among premenopausal women, those with 25OHD concentrations above the median had significantly reduced odds of grade III cancer (OR = 0.46, 95% CI = 0.29–0.74), ER negative cancer (OR = 0.34, 95% CI = 0.17–0.66), and triple negative cancer (OR = 0.21, 95% CI = 0.08–0.53). Using continuous vitamin D data, an incremental increase of 10 ng/mL 25OHD concentrations was associated with about two thirds reduction of odds of triple negative breast cancer (OR = 0.36, 95% CI = 0.22–0.56) (Figure S1). Among postmenopausal women, higher serum vitamin D levels were associated with reduced odds of breast cancer regardless of tumor characteristics.

In case-series analyses, high levels of serum 25OHD were less likely to be associated with premenopausal breast cancer with poor prognostic characteristics than low levels (grade III versus I/II, OR = 0.45, 95% CI = 0.22–0.91; ER negative versus positive, OR = 0.48, 95% CI = 0.21–0.93; triple negative versus luminal A subtype, OR = 0.26, 95% CI = 0.09–0.71) (Figure 2). Similar results were also found with a 10 ng/mL incremental increase of serum 25OHD concentrations (Figure S2). In contrast, there were no associations of 25OHD levels with cancer prognostic characteristics in parallel analyses among postmenopausal women (Figures 2 and S2).

Discussion

We found that higher serum 25OHD concentrations were associated with significantly reduced odds of both premenopausal and postmenopausal breast cancer. Among premenopausal women only, 25OHD concentrations were significantly lower in women with tumors with poor prognostic characteristics (high grade, ER negative, and triple negative) than among those with cancers with better prognostic features. The findings support the hypothesis that vitamin D may reduce risk of the development of a subset of tumors with more aggressive characteristics and poorer prognosis.

Existing evidence supports a link between vitamin D and prognostic characteristics of breast cancer. In clinical studies, serum 25OHD concentrations have been inversely associated with breast cancer stage [23,24] and histologic grade [25]. In a multiethnic cohort of breast cancer survivors, women with ER negative breast cancer had significantly lower serum 25OHD than those with ER positive tumors [24], and in a case-control study with both pre- and postmenopausal women, reduced risk of breast cancer with higher 25OHD levels was found only among women with ER-/PR- tumors [26]. In a German case-control study of premenopausal women, plasma 25OHD and dietary vitamin D intake were more strongly related to ER- or PR- breast cancer

Table 2. Odds ratios and 95% confidence intervals for breast cancer by serum 25-hydroxyvitamin D levels.

Serum 25OHD levels	All			Premenopausal			Postmenopausal		
	case n (%)	control n (%)	OR (95% CI)	case n (%)	control n (%)	OR (95% CI)	case n (%)	control n (%)	OR (95% CI)
Deficient	220 (38)	156 (27)	1.00	82 (33)	74 (30)	1.00	138 (41)	82 (25)	1.00
Insufficient	241 (42)	203 (35)	0.81 (0.61–1.08)	110 (45)	83 (34)	1.13 (0.72–1.77)	131 (39)	120 (36)	0.64 (0.44–0.94)
Sufficient	118 (20)	215 (37)	0.37 (0.27–0.51)	53 (22)	88 (36)	0.57 (0.34–0.93)	65 (19)	127 (39)	0.29 (0.19–0.45)
P-value for trend			<0.001			0.03			<0.001
Continuous per 10 ng/mL increment	579	574	0.67 (0.59–0.75)	245	245	0.76 (0.63–0.91)	334	329	0.61 (0.52–0.72)

[1]Serum 25-hydroxyvitamin D (25OHD) concentrations were adjusted by the week of blood collection time in a year by locally weighted multinomial regression. The three levels were defined as follows: deficient, <20.0 ng/mL; insufficient, 20.0–29.9 ng/mL; sufficient, ≥30.0 ng/mL.

[2]Odds ratios (OR) ad 95% confidence intervals (CI) were adjusted for age and BMI. Further adjustment for physical activity did not significantly change the results (data not shown).

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Table 3. Serum 25-hydroxyvitamin D concentrations by prognostic characteristics in premenopausal and postmenopausal women diagnosed with breast cancer.

Tumor characteristics	All (n = 579)	Premenopausal women (n = 245)			Postmenopausal women (n = 334)		
	N (%)[1]	N (%)[1]	mean ± se[2], ng/mL	P-value	N (%)[1]	mean ± se[2], ng/mL	P-value
Tumor stage				<0.001			0.23
In situ	86 (15)	42 (17)	28.9±1.4		44 (13)	24.8±1.4	
I	292 (51)	95 (39)	24.8±0.9		197 (59)	22.3±0.7	
II/IIIA	179 (31)	96 (39)	21.3±1.0		83 (25)	21.4±1.0	
IIIB/IIIC/IV	20 (3)	11 (5)	20.0±2.7		9 (3)	24.4±3.0	
Histologic grade				0.005			0.81
I/II	166 (35)	56 (29)	26.0±1.3		110 (40)	21.9±0.8	
III	305 (65)	137 (71)	21.6±0.8		168 (60)	22.1±0.7	
ER status				0.03			0.76
Positive	372 (76)	147 (73)	23.7±0.8		225 (79)	22.1±0.6	
Negative	115 (24)	55 (27)	20.2±1.3		60 (21)	21.7±1.2	
Molecular subtype				0.002			0.92
Luminal A	330 (69)	129 (64)	24.5±0.8		201 (71)	22.2±0.6	
Luminal B	49 (10)	23 (11)	21.2±1.9		26 (9)	21.1±1.7	
Non-luminal HER2+	32 (6)	15 (7)	21.7±2.5		17 (6)	21.2±2.2	
Triple negative	74 (15)	34 (17)	17.5±1.6		40 (14)	21.8±1.4	

[1]Two patients with tumor stage not evaluable (TX) were excluded from analysis of stage. For the analysis of histologic grade, ER status and molecular subtype, women with carcinoma *in situ* (n = 86) were excluded. The numbers do not add up to the total due to missing data: histologic grade (missing n = 22 or 4%), ER status (missing n = 6 or 1%), and molecular subtype (missing n = 8 or 2%).
[2]Least square mean and standard error (se) were adjusted for age, season of blood collection, and body mass index in linear regression models. Additional adjustment for physical activity did not significantly change the results (data not shown).
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than hormone receptor (HR) positive cancers [27,28]. Similar to our findings, among postmenopausal women from the same study, the inverse relationships between 25OHD levels and breast cancer risk did not differ by HR status [29]. However, there are also studies with observed associations only for HR positive cancers [30,31] or with null findings [32,33].

The inconsistency in results across studies could be explained by heterogeneity in study populations, in classification of HR status, and/or in assessment of vitamin D status (dietary intake versus circulating 25OHD). Our patient population represents a more contemporary patient cohort (2003–2008) with data available on ER, PR and HER2, allowing us to refine tumor subtype classification and to distinguish the triple negative subtype. However, we were not able to classify the basal-like subtype due to lack of data on basal markers CK5/6 or EGFR. Although basal-like and triple negative phenotypes largely overlap and share a poor prognosis, the former definition represents a more refined group by excluding the unclassified subtype, which may behave differently in prognosis from the basal-like subtype. Our findings warrant validation in large prospective studies where complete data on molecular subtypes are available.

Although the exact biological mechanisms are not clear, data from animal experiments are concordant with our findings. *Vdr* knockout mice gavaged with the carcinogen dimethylbenzanthracene (DMBA) were more likely to develop ER-/PR- mammary tumors than wild type littermates [34]. Moreover, *VDR* expression were remarkably lower in ER- than in ER+ breast tumors [35], and the elevation of VDR nuclear corepressor NCoR1 level was particularly associated with ER negativity [36]. There are two possible explanations for these findings. First, vitamin D may prevent the occurrence of ER negative breast cancer by interfering with estrogen signaling pathway, as treatment with $1,25(OH)_2D$ down-regulated the abundance of ER and suppressed estrogen activity in breast cancer cells [37], and vitamin D supplementation significantly reduced blood levels of progesterone and estradiol in women [38]. Second, vitamin D may prevent aggressive breast cancers by modulating the extracellular microenvironment, as vitamin D has been shown to alter the expression of a variety genes involved in extracelluar matrix remodeling [39,40] and to modulate breast cancer phenotypes [41].

A limitation of our study is that only a single measurement of vitamin D at diagnosis was used, which may not necessarily represent vitamin D levels at the time of cancer initiation or progression. However, in a recent study, the correlation coefficient for measurement of 25OHD concentrations in serum samples collected in 1994 and 2008 ranged from 0.42 to 0.52, and was 0.80 when measured 12 months apart [41], suggesting reasonable stability of endogenous vitamin D status. Because blood samples in our study were collected shortly after diagnosis, prior to surgery or any adjuvant therapy, there would be little influence on vitamin D levels from life style changes after cancer diagnosis or from treatment.

In conclusion, our study provides compelling evidence that endogenous vitamin D levels may be associated with the etiology of breast cancer, particularly the triple negative subtype leading to poor prognosis among premenopausal women. Because the risk of triple negative breast cancer peaks before menopause, and because vitamin D deficiency can be easily corrected by increasing sun exposure and/or supplement intake, if our findings are confirmed in large prospective studies for temporal causality, vitamin D may be used as a potential cancer preventive agent against triple negative cancers among young women.

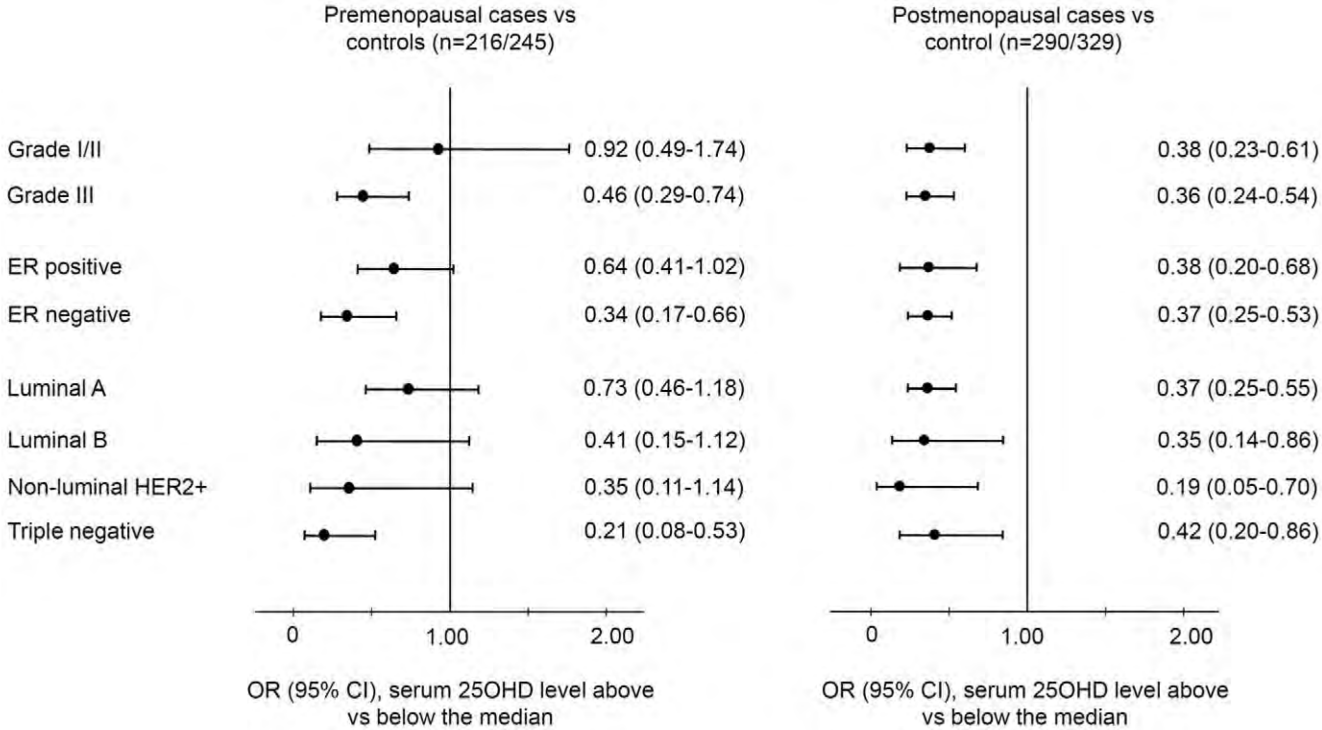

Figure 1. Case-control analysis of breast cancer risk by high and low vitamin D levels. Season-standardized serum 25-hydroxyvitamin D (25OHD) concentrations were stratified into above and below the median in healthy controls by menopausal status. Odds ratios (OR) and 95% confidence intervals (CI) were derived from multinomial logistic regression with adjustment for age at diagnosis and BMI, and presented in groups of tumor characteristics, where healthy controls were used as a referent group. Further adjustment for physical activity did not significantly change the results (data not shown). The lengths of horizontal lines are indicative of confidence intervals and the dots are indicative of odds ratios, with the corresponding odds ratios and 95% confidence interval given in numbers on the right of the Y-axis.
doi:10.1371/journal.pone.0017251.g001

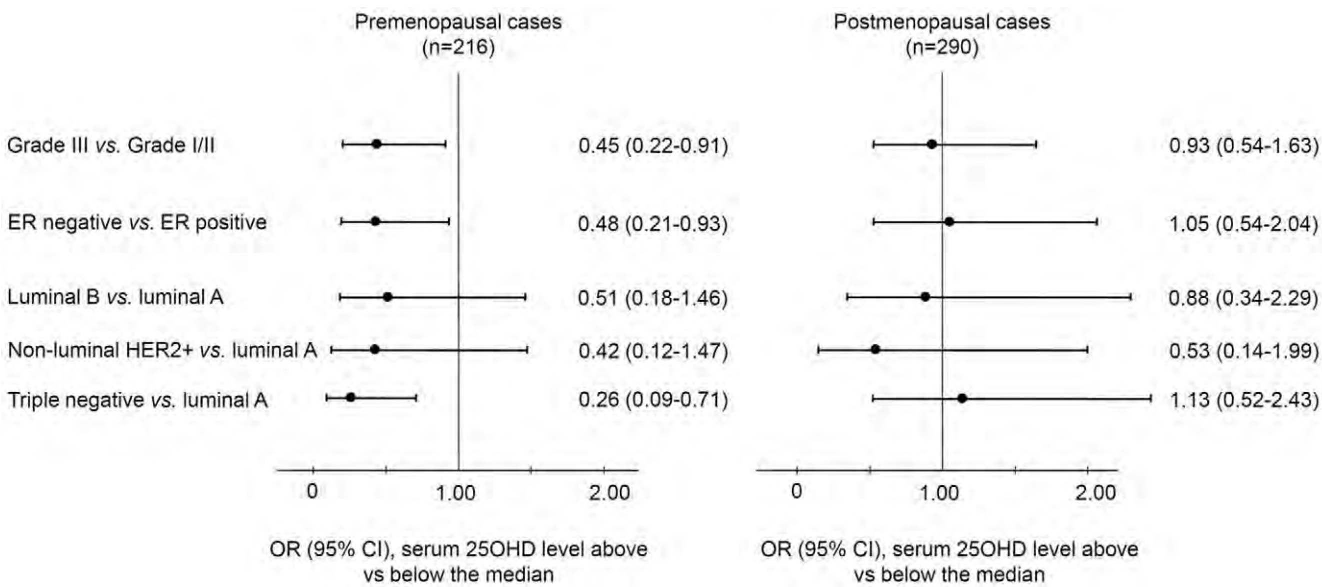

Figure 2. Case-series analysis of breast cancer risk by high and low vitamin D levels. Season-standardized serum 25-hydroxyvitamin D (25OHD) concentrations were stratified into above and below the median in healthy controls by menopausal status. Odds ratios (OR) and 95% confidence intervals (CI) were derived from multinomial logistic regression with adjustment for age at diagnosis and BMI, and presented in groups of tumor characteristics, where women with better prognostic characteristics (grade I/II, ER+, or luminal A subtype) were used as a referent group and women with carcinoma *in situ* (CIS) were excluded. Further adjustment for physical activity did not significantly change the results (data not shown). The lengths of horizontal lines are indicative of confidence intervals and the dots are indicative of odds ratios, with the corresponding odds ratios and 95% confidence interval given in numbers on the right of the Y-axis.
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Supporting Information

Figure S1 Case-control analysis of breast cancer risk by 10 ng/ml increase of vitamin D levels. Season-standardized serum 25-hydroxyvitamin D (25OHD) concentrations were entered into the regression models as a continuous variable. Odds ratios (OR) and 95% confidence intervals (CI) of an incremental increase of 10 ng/mL 25OHD were derived from multinomial logistic regression with adjustment for age at diagnosis and BMI, and presented in groups of tumor characteristics, where healthy controls were used as a referent group. Further adjustment for physical activity did not significantly change the results (data not shown). The lengths of horizontal lines are indicative of confidence intervals and the dots are indicative of odds ratios, with the corresponding odds ratios and 95% confidence interval given in numbers on the right of the Y-axis.
(TIF)

Figure S2 Case-only analysis of breast cancer risk by 10 ng/ml increase of vitamin D levels. Season-standardized serum 25-hydroxyvitamin D (25OHD) concentrations were entered into the regression models as a continuous variable. Odds ratios (OR) and 95% confidence intervals (CI) of an incremental increase of 10 ng/mL 25OHD were derived from multinomial logistic regression with adjustment for age at diagnosis and BMI, and presented in groups of tumor characteristics, where women with better prognostic characteristics (grade I/II, ER+, or luminal A subtype) were used as a referent group and women with carcinoma *in situ* (CIS) were excluded. Further adjustment for physical activity did not significantly change the results (data not shown). The lengths of horizontal lines are indicative of confidence intervals and the dots are indicative of odds ratios, with the corresponding odds ratios and 95% confidence interval given in numbers on the right of the Y-axis.
(TIF)

Author Contributions

Conceived and designed the experiments: SY LES AEM CSJ CBA. Performed the experiments: SY MKN WD CBA. Analyzed the data: SY LES AEM CSJ CCH SEM CBA. Contributed reagents/materials/analysis tools: MKN WD HW SK SBE TOC CBA. Wrote the manuscript: SY LES AEM CSJ DLT MKN WD CCH SEM HW SK SBE TOC CBA. Financial support: CBA.

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